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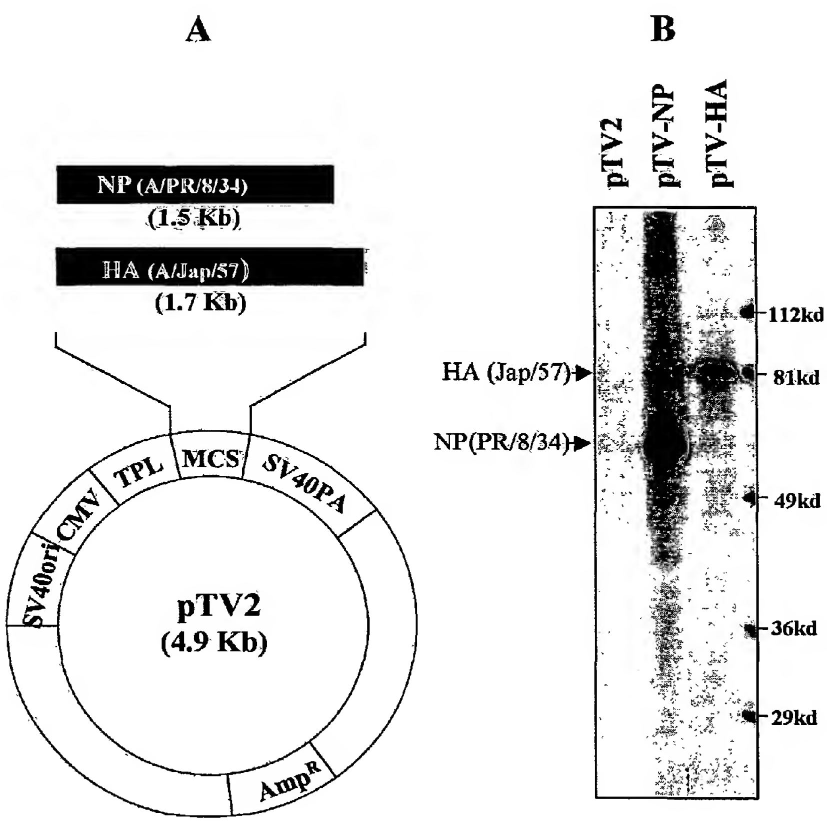
(54) **Title:** METHOD FOR ENHANCING IMMUNE RESPONSES BY CODELIVERING INFLUENZA NP DNA IN DNA IMMUNIZATION



(57) Abstract: The present invention relates to the use of influenza NP (nucleoprotein) DNA as an adjuvant for enhancing immune responses to a DNA vaccine, in particular, a method for enhancing immune responses by codelivering influenza NP DNA and DNA vaccine against an immune antigen, and a vaccine composition comprising said influenza NP DNA and said DNA vaccine for the immune antigen. According to the method of the present invention, the influenza NP gene DNA is used as an adjuvant for a DNA vaccine to enhance the immune response of the DNA vaccine such that it can be used for effective prevention against or treatment of influenza, AIDS, hepatitis B, hepatitis C, cancer, tuberculosis, malaria, etc. and to assist in the development of influenza vaccines.

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Method for enhancing immune responses by codelivering influenza NP DNA in DNA immunization

FIELD OF THE INVENTION

The present invention relates to the use of influenza NP (nucleoprotein) DNA as an adjuvant for enhancing immune responses to a coimmunized DNA. The present invention relates, in particular, to a method for enhancing immune responses by codelivering an influenza NP DNA and a DNA vaccine against an antigen, and a vaccine composition comprising said influenza NP DNA and said DNA vaccine for the antigen.

BACKGROUND OF THE INVNETION

In recent, methods have been developed that can prevent infectious diseases such as influenza by DNA immunization. It has been reported that, when immunizing animals with DNA of nucleoprotein (hereinafter, referred to as 'NP') or hemagglutinin (hereinafter, referred to as 'HA') or matrix (M1, M2) gene of influenza virus by intramuscular injection, gene gun, etc., can elicit more enhanced protective effect against influenza viral infection (Ulmer JB, *Science* 259:1745, Robinson HL, *Vaccine* 11:957, Okuda K, *Vaccine* 19:3681). In this regard, it has also been known that the neutralizing antibody response and cellular immune response, including CTL (Cytotoxic T lymphocyte) response against an antigen, induced by DNA immunization, are related to the extent of the protective effect against viral infection (Fu TM, *J.*

Immunol. 162:4163, Ulmer, *J. Virol.* 72:5648, Robinson HL, *J. Infect. Dis.* 176:S50). Protection against a specific influenza viral infection in mice model can be induced by DNA immunization with one type of NP gene (Ulmer JB, *Science,* 259:1745), HA (Robinson HL, *Vaccine*, 11:957), matrix (Okuda K, *Vaccine*, 19:3681) and neuraminidase (Chen Z, *Vaccine*, 18:3214), being genes of influenza viruses; however, it has been found that B-type viral infection cannot be prevented even by immunization with homogeneous NP DNA (Chen Z, *Vaccine*, 19:1446), which means that immunization with the mixture of various types of influenza viruses is necessary for protection against the various types of influenza viruses.

Immunization with the DNA mixtures of genes of several antigens is required in some cases: (1) where the immune responses against several antigens are indispensable for obtaining the protective effect against one type of viral infection (Chen Z, *Vaccine*, 17:653; Bot A, *Vaccine*, 16:1675), (2) where for protection from a virus with a rapid mutation property, it is necessary to immunize with DNA encoding its various mutant antigens at the same time (Lu S., *J. Virol.* 70:3978), and (3) where it is required to obtain simultaneously and simply the immune responses against more than two types of viruses.

Multivalent DNA vaccine technology for coimmunization with DNA encoding genes of several antigens has been used for DNA immunization against several viruses such as HIV (Amara RR, *Science*, 292:69), SIV (Lu S. *J. Virol.* 70:3978), HBV (Musacchiro A, *BBRC*, 282:442), etc. For influenza DNA immunization, NP DNA + HA DNA immunization, NA DNA + HA DNA immunization and the like have also been tried and these coimmunizations were confirmed to exhibit a higher protective efficiency, as compared with immunization with DNA of one type antigen alone (Chen Z, *Vaccine*, 17:653; Bot A, *Vaccine*, 16:1675). However, it is unclear whether coimmunization with the mixture of influenza DNA vaccines causes any difference in the cellular immune response or antibody formation, induced by each DNA vaccine, as compared with the immunization with one-type DNA vaccine alone. The report that

upon immunization with the mixture of one type DNA and another type DNA, as one type of DNA is replaced, the ratio of IgG1/IgG2a produced may be changed (Braun R, *J. Gen. Virol.* 79:2965), and the report that in coimmunization with HIV rev gene and rev gene, T cell immune response can be inhibited, as compared with immunization with one type of gene alone (Kjerrstorm A., *Virology*, 284:46), suggest that one type of DNA can have an effect on the immune response induced by other type of DNA.

Meanwhile, for the purpose of enhancing antibody response, cellular immune response and protective effect, which are induced by DNA immunization, use of various genetic adjuvants has been tried. It has been known that use of cytokine genes such as IL-2 (Lee et al, *Vaccine*, 17:473), GMCSF (Lee et al, *J. Virol.* 72:8430; Cho et al, *Vaccine*, 17:1136) and IL-12 (Kim JJ et al, *J. Immunol.* 158:816), and codelivery of a synchronic stimulating molecular gene such as CD40L (Gurunathan S et al., *J. Immunol.* 161:4563; Mendoza RB et al, 159:5777), ICAM-1(Kim JJ et al., *J. Clin. Invest.* 103:869), B7-1 and B7-2 (Tsuji T et al., *Eur. J. Immunol.* 27:782; Kim JJ et al., *Nat. Biotechnol.* 15:641) enhance the induction of immune response. Moreover, it has been shown that fusion of a ubiquitin gene (Rodriguez F et al., *J. Virol.* 71:8497) and incorporation of a specific DNA sequence such as CpG motif enhance the immune response against an antigen (Sato Y et al., *Science,* 273:352). However, it has never been shown that one of components comprising a vaccine has an effect as an adjuvant on the immune response to a coimmunized DNA vaccine.

The inventors of the present invention found that, when administering a DNA vaccine together with an influenza NP gene DNA, the antibody response and CTL response to the DNA vaccine are enhanced and the secretion of IFN-γ increases, as compared with administration of the DNA vaccine alone, such that the antibody immune response induced by the DNA vaccine can be enhanced. The present invention was accomplished based upon the above novel fact.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for enhancing an immune response by administering a DNA vaccine against an antigen (sometimes, referred to as "immune antigen") together with an influenza NP gene DNA, and a vaccine composition comprising said DNA vaccine against the immune antigen and said influenza NP gene DNA.

In order to accomplish the above object, the present invention provides the method for enhancing the immune response by administering the DNA vaccine against the immune antigen together with the influenza NP gene DNA. Furthermore, the present invention provides the vaccine composition comprising said DNA vaccine against the immune antigen and said influenza NP gene DNA.

Herein below, the present invention will be described in more detail.

The present invention provides use of said influenza NP gene DNA as an adjuvant to enhance an immune response.

The influenza NP gene DNA in the present invention is DNA having a nucleotide sequence for encoding a protein having more than 90% amino acid sequence homology to an influenza NP protein. Preferably, the full or partial region of said DNA is used for the present invention. More preferably, the full or partial region of DNA having the nucleotide sequence described in "Sequence No. 1" is used for the present invention. Where the partial region of influenza NP gene is used, the same effect can be exhibited under the condition that said DNA has a nucleotide sequence for encoding the amino acid sequence which includes more than the N-terminal 50% or more than the C-terminal 50% of influenza NP protein,

The influenza NP gene DNA of the present invention is administered together with a DNA vaccine, preferably, with the influenza NP gene DNA inserted into an

expression vector. The expression vector for insertion of said influenza NP gene DNA thereinto is, preferably, a vector having the transcription promotor selected from a group consisting of CMV (cytomegalovirus) promotor, RSV (Rous sarcoma virus) promotor, β-actin promotor, SV40 (simian virus 40) promotor and muscle creatine kinase promotor, and the transcription terminator selected from a group consisting of SV40 poly(A) and BGH terminator; more preferably, an expression vector having the early promotor / enhancer sequence of cytomegalovirus and the adenovirus tripartite leader / intron sequence and containing the replication orgin and poly(A) sequence of SV40.

The inventors of the present invention created pTV-NP by inserting the influenza NP gene DNA, having the nucleotide sequence of Sequence No. 1, into pTV 2 expression vector, and transformed *Escherichia coli* with said pTV-NP. This transformant, named as "XL1-blue/pTV-NP", was deposited in Gene Bank (located in Korea Research Institute of Bioscience and Biotechnology) under the accession No. KCTC 10193BP on February 27, 2002.

The DNA vaccine of the present invention is administered together with the influenza NP gene DNA, upon inoculation of the DNA vaccine against one or more immune antigens selected from a group consisting of influenza, varicella virus, diphtheria, tetanus, polio virus, malaria, herpes virus, HIV, papilloma virus, hepatitis B virus, hepatitis C virus, rotavirus, cholera, measles and tuberculosis. This coimmunization enhances the antigen-specific antibody response and CTL (cytotoxic T lymthocytes) response and increases the secretion of IFN-γ to enhance Th-l (helper T cell) such that the immune response induced by the DNA vaccine is enhanced.

In a preferred embodiment according to the present invention, the influenza NP gene DNA having the nucleotide sequence, described in Sequence No. 1, was inserted into pTV2 vector to prepare pTV-NP, and then the immune response induced by the influenza NP gene DNA was tested.

As a result of testing, coimmunization with influenza HA DNA and influenza NP gene DNA (hereinafter, referred to as 'NP DNA'), according to the present invention, did not change the immune response specific to NP DNA *per se*, as compared with immunization with NP DNA alone; however, this coimmunization enhanced formation of the antibodies specific to HA and Th-1 (helper T cell 1) and CTL response, as compared with immunization of HA DNA alone (refer to FIG. 2). Accordingly, it can be understood that the antibody response specific to HA increases due to several effects caused by coimmunization. Immunization with even a small amount of NP DNA can induce a strong antibody response and CTL response for a short time, which was first found by the inventors of the present invention and other researchers (Lee SW, *Immunology* 94:285). It is inferred that the strong helper T cell response, induced by NP DNA immunization, leads to activation or production of a factor, being capable for activating a nonspecific immune response such as cytokine secretion, whereby the immune response specific to HA (antibody, CD4 and CD8 responses) is enhanced. In the recently that the V3-specific antibody response and CTL response are enhanced as compared with immunization with MNgp160 DNA when MN V3 region of HIV-1 gene is fused with HBV surface antigen (Fomsgaard A Scand. *J. Immunol.* 47:289). NP DNA in the present invention can be understood to have the function of a genetic carrier, similar to HBV S gene, above. In contrast, immunization with HA DNA had no effect on the NP-specific immune response, which may be because a specific immune response induced by NP DNA is relatively stronger than the HA-specific immune response induced by HA DNA. As a result, coimmunization with NP DNA enhances the antibody immune response induced by the other DNA vaccine, being simultaneously administered, while not enhancing the NP-specific antibody response induced by NP DNA *per se*.

Moreover, increase of the immune response specific to another antigen, by coimmunization with NP DNA, also takes place in cases HCV E2 DNA and HIV env DNA as well as HA DNA (refer to FIG. 3). Accordingly, NP DNA can be used as a

genetic immune response adjuvant for a DNA vaccine against influenza, HIV, hepatitis C virus, measles, tuberculosis, etc. Furthermore, coimmunized viruses, which NP DNA can be applied as an immune response adjuvant to, are not limited to the above ones but include the ones with properties similar to the above viruses.

The concentration of IFN-γ secreted from the lymphocytes of mice, which were immunized with NP DNA or HA DNA, was measured and, as a result, the amount of secreted IFN- γ was confirmed to increase by coimmunization with NP DNA (refer to FIG. 4). The above result shows that coimmunization with a DNA vaccine and NP DNA increases Th-1 response as well as antibody response and CTL response.

In another experiment for confirming whether NP DNA of the present invention can also be used as an adjuvant for enhancing the immune response to other types of coimmunized antigens, e.g., OVA (ovalbumin), in addition to HA, HIV env and HCT E2, simultaneous administration of OVA DNA and NP DNA increased the amount of OVA-specific IFN-γ producing T-cells, as compared with the case of administering OVA DNA alone (refer to FIG. 5). The above result shows that the effect of NP DNA as an immune response adjuvant can be applied to a relatively broad range of antigens to increase CD4 and CD8 T cell response.

The adjuvant effect of NP DNA on the immune response can also be directly observed *in vivo*; i.e., CD4 or CD8 T cellular response, induced by other antigens such as HA, Env, E2 etc., administered together with NP DNA, in particular, the rate of cell division was observed to increase. Moreover, OT-II cell division, which was not observed upon administration of OVA DNA alone, could be remarkably induced when OVA DNA was administered together with NP DNA (refer to FIG. 6). Considering a previous report that OT-II cells need about 100 times more antigen than do OT-I cells so as to be induced to divide *in vitro* or *in vivo* (Ming L et al., *J. Immunol.* 2001, 166:6099), the above result seems to suggest that NP DNA has an effect on the OVA-specific T cell division, induced by OVA DNA, by controlling the activation threshold

for T cell activation. Furthermore, the previous report showed that action of CD4 T cells is important for the formation and intensity of CTL response and it can thus be understood that the OVA-specific CD4T cell response, increased by NP DNA, has a positive effect on the formation and intensity of CD8 T cell response.

Whereas the NP DNA immunization increases the specific immune response to a coimmunized DNA, immunization with the mixture of HIV env DNA and HCV E2 DNA may decrease the CTL response induced by each DNA. The E2-specific CTL response showed lysis values of 10.6% and 13.6%, respectively, in two separate analyses, upon coimmunization with env DNA and E2 DNA, but 45.1% and 33.4% upon immunization with E2 DNA alone. Also, the env-specific CTL response was lower upon coimmunization with env DNA and E2 DNA (4.1%, 0.2%) than upon immunization with env DNA (26.7%, 18.3%) alone. Accordingly, upon coimmunization with the mixture of two types of antigen DNAs, increase of the immune response specific to one type of antigen depends upon the kind of antigen, and occurs only upon using the gene of a specific antigen, such as NP DNA.

The NP DNA + HA DNA immunization increases the initial survival rate and mean body weight upon infection with the lethal dose of viruses, as compared with NDNA immunization or HA DNA immunization alone (refer to FIG. 8). According to a previously disclosed report (Bot A Vaccine 16:1675), when mice were immunized with the mixture of NP DNA (PR/8) vaccine and HA DNA (WSN) vaccine and then challenged with influenza virus strain WSN, the survival rate of mice increased, as compared with immunization with one of HA DNA and NP DNA. However, the above report also showed that, upon challenging mice in the same group with PR/8 virus, the survival rate of mice did not increase. Although the HA-specific antibody response or NP-specific CTL response has been known to be related to prevention against the infection of influenza virus (Ulmer JB, *J. Virol.* 72:5648, Robinson HL, *J Infect Dis.* 176:S50-5), the intensities of immune response necessary for increasing the survival rate may be different, depending upon the types of influenza viruses to be challenged.

Using NP DNA as an adjuvant for enhancing an immune response to a coimmunized DNA in the present invention will assist in development of influenza vaccines, and also assist research on immune response adjuvants for AIDS and Hepatitis vaccine and research for efficiency enhancement of a vaccine comprising two or more types of DNAs. The system used in the present invention can be applied to the research models of immune interference or enhancement in DNA immunization with multiple-components.

Furthermore, the present invention provides a vaccine composition comprising an influenza NP gene DNA and a DNA vaccine against an immune antigen.

The Influenza NP gene DNA in the present invention is DNA having a nucleotide sequence for encoding a protein having more than 90% amino acid sequence homology to the influenza NP protein. The full region of said DNA or a partial region having more than 50% of the full region can be used for the vaccine composition of the present invention. More preferably, the full region of DNA having the nucleotide sequence described in "Sequence No. 1" or a partial region having more than 50% thereof can be used for the vaccine composition of the present invention.

In the vaccine composition of the present invention, the influenza NP gene DNA is preferably used in a form inserted into an expression vector. The expression vector for insertion of said influenza NP gene DNA thereinto is, preferably, a vector having the transcription promotor selected from a group consisting of CMV (cytomegalovirus) promotor, RSV (Rous sarcoma virus) promotor, β-actin promotor, SV40 (simian virus 40) promotor and muscle creatine kinase promotor, and the transcription terminator selected from a group consisting of SV40 poly(A) and BGH terminator; more preferably, a vector having the early promotor/enhancer sequence of cytomegalovirus and the adenovirus tripartite leader/intron sequence, and containing the replication orgin and poly(A) sequence of SV40; especially preferably, pTV-NP with the influenza NP gene DNA, having the nucleotide sequence of Sequence No. 1,

inserted into pTV 2 expression vector.

The DNA vaccine in the vaccine composition of the present invention is a DNA vaccine against one or more immune antigens selected from a group consisting of influenza, varicella virus, diphtheria, tetanus, polio virus, malaria, herpes virus, HIV, papilloma virus, hepatitis B virus, hepatitis C virus, rotavirus, cholera, measles and tuberculosis; however, said DNA vaccine is not limited to the above ones.

One of advantages of DNA immunization is that the formulation process is simple for simultaneously obtaining immune responses against several antigens; i.e., the immune responses against several antigens can be obtained by simply mixing DNA vectors having the gene of each antigen. Accordingly, the vaccine composition of the present invention contains 1 – 99% by weight, preferably, 25 – 60% by weight of said influenza NP gene DNA on the basis of the total weight of composition; however, it is not limited to this range and can be changed, depending upon the type of DNA vaccine, and a patient's condition.

For the purpose of enhancing the stability of antigen, soluble excipients can be contained into the vaccine composition of the present invention, in addition to said NP DNA and DNA vaccine, which examples of these excipients include carbohydrate, amino acid, fatty acid, inorganic salt, surfactant, polyethylene glycol, mixtures of these, etc. Representative examples of said carbohydrate include soluble sugars such as hydropropyl cellulose, carboxymethyl cellulose, sodium carboxyl cellulose, hyaluronic acid, chitosan, alginate, glucose, xylose, galactose, fructose, maltose, saccharose, dextran, chondroitin sulfate, etc. Representative examples of said protein include albumin, gelatin, etc. Representative examples of said amino acid include glycine, alanine, glutamic acid, arginine, lysine, and their salts.

The vaccine composition of the present invention may be filled with ones selected from a group consisting distilled water, saline solution and PBS (phosphate buffered saline) with influenza NP gene DNA and DNA vaccine.

The vaccine composition of the present invention follows general administration and formulation methods of DNA vaccine (Wolff et al., *Science,* 1990, 247:1465). DNA vaccine can be delivered to exodermis by the direct injection, particle impact or electroporation method, or be delivered to exodermis by the complex and/or repeated administration method. DNA vaccine can also be administered by coating DNA onto a gold bead and then delivering the gold bead into cells using a gene gun (Porgador et al., *The Journal of Experimental Medicine*, 1998, 188:1075). In addition, the vaccine composition of the present invention can be formulated as an oral drug or non-oral drug by general methods and then administered via an oral or non-oral route. For formulation of drug, diluents or excipients such as filler, extender, coupler, wetting agent, disintegrant, surfactant and the like are generally used. Solid drugs for oral administration include tablet, pill, powder, granule, capsule, etc. These solid drugs can be prepared by mixing more than one excipient such as calcium carbonate, sucrose or lactose, gelatin, etc. In addition to these excipients, lubricants such as magnesium stylate talc can also be used. Liquid drugs for oral administration include suspension, solution, emulsion, syrup, etc., and can contain several general excipients such as wetting agent, sweet agent, aromatic agent, preservative in addition to diluents such as water or liquid paraffin. Drugs for non-oral administration include sterile solution, non-soluble solution, suspension, emulsion, lyophilized drug, suppository, etc. For non-soluble or suspension drugs, used can be vegetable oil such as propylene glycol, polyethylene glycol, olive oil, etc. and injectable ester such as ethyl olerate. For carriers of a suppository, used can be witepsol, macrogol, tween 61, cacao butter, lauric fat, glycerol gelatin, etc. In particular, where the composition of the present invention is administered into mucous membrane (nasal mucous membrane), the composition for administration can be formulated to liquid or dry powder capable of being administered in the form of aerosol spray.

The administration amount and number of the vaccine composition of the present invention can be determined by the proper administration amount and number,

known for an antigen as used, and can be changed depending upon body weight, age, sex, health condition, food, administration time, administration method, excretion rate, extent of illness, etc.

The effective amount of Influenza NP gene DNA, contained in the composition of the present invention, is 10 μg – 10 mg/kg by body weight and, preferably, 100 μg – 5 mg/kg by body weight. Furthermore, the vaccine composition of the present invention is not toxic and thus very safe as a DNA vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1(A) is a genetic map of vector, illustrating regions where NP gene DNA (NP DNA: 1.5 kb) and HA DNA (1.7 kb) are inserted into pTV2 vector, and FIG. 1(B) is photographs taken by the radioimmunoprecipitation method, when having expressed pTV-NP and pTV-HA in COS-7 cells.

FIG. 2(A) is graphs of the antibody response analyzed by conducting ELISA with HA-specific antibody or NP-specific antibody, when having immunized mice with NP DNA, HA DNA, or NP DNA + HP DNA, FIG. 2(B) is graphs of HA-specific or NP-specific CTL response analyzed when having immunized mice with NP DNA, HA DNA, or NP DNA + HA DNA.

FIGS. 3(A) and 3(B) are graphs of the antibody response analyzed by conducting ELISA with HIV-specific antibody, E2-specific antibody, or NP-specific antibody, when having coimmunized mice with env or E2 DNA and NP DNA, FIGS. 3(C) and 3(D) are graphs of env-specific CTL, E2-specific CTL or NP-specific CTL response analyzed when having coimmunized mice with env or E2 DNA and NP DNA.

FIG. 4 is a graph of the IFN-γ concentration, secreted from HA-stimulated lymphocyte, analyzed by ELISA, when having immunized mice with HA DNA, NP

DNA, HA DNA + NP DNA, or DNA.

FIG. 5 is graphs of the number of IFN-γ secreting cells, by NP peptide, OVA257-264 peptide, or OVA323-339 peptide, analyzed by ELISPOT, when having immunized mice with NP DNA.

FIG. 6 is photographs of showing proliferation of CFSE-labeled OT-I cells, analyzed by a laticiferous cell analyzer, when having immunized mice with NP DNA + OVA DNA, OVA DNA, or NP DNA.

FIG. 7 is photographs of showing proliferation of CFSE-labeled OT-II cells, analyzed by the laticiferous cell analyzer, when having coimmunized mice with NP DNA + OVA DNA, OVA DNA, or NP DNA.

FIG. 8 is graphs of illustrating the change of survival rate (A) and mean body weight (B), when having challenged mice with the lethal influenza dose after immunization with NP DNA + HA DNA, NP DNA, HA DNA, or control DNA.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is described in more detail with reference to the following examples. However, the scope of the present invention is not limited to these.

EXAMPLE 1: Preparation of DNA vaccine

The inventors of the present invention obtained the desired genes from viruses to prepare a DNA vaccine. Specifically, MDCK cells were infected with Influenza A/PR/8/34 and Influenza A/Jap/57 viruses, respectively, and total RNA was isolated. Then, the NP gene of Influenza A/PR/8/34 (Genebank accession No. M38279), having the nucleotide sequence of Sequence No. 1, and the HA gene of Influenza A/Jap/57

(Genebank accession No. L20407), having the nucleotide sequence of Sequence No. 2, were obtained by conducting RT-PCR with the isolated RNA. Theses NP and HA genes cleaved by XhoI / XbaI and KpnI / XhoI were inserted into pTV2 vector (Lee SW, *J. Virol.* 72:8430) to prepare pTV-NP and pTV-HA (refer to FIG. 1A), respectively. The pTV vector has the early promotor/enhancer sequence of Cytomegalovirus and the adenovirus tripartite leader/intron sequence and contains the replication orgin and polyA sequence of SV40.

Meanwhile, pTX GE (Lee AH et al., *Vaccine*, 17:473) containing the HIV env gene was cleaved by MluI and HpaI, and inserted into pTV2 vector to prepare pTV-GE. For HCV E2 DNA, pTV-gDs-E2t (Lee SW, *J. Virol.* 72:8430) was used without any modification. Chicken OVA (ovalbumin) cDNA from Tc-OVA vector was amplified by PCR and inserted into pTV2 vector to prepare pTV-OVA. Each DNA vaccine was grown in *E. coli* and then purified using endotoxin-free kit (QIAGEN).

The DNA vaccine as prepared above was confirmed by transient transfection assay. Specifically, 3×10^5 COS-7 cells were transfected with 10 μg of pTV-NP, pTV-HA or pTV-OVA by the calcium phosphate method. Forty-eight hours after transfection, the cells were labeled with 32S-Met for 12 hours and harvested. The expression of NP and HA proteins was confirmed using anti-Flu (PR/8/34) mouse serum by the radioimmunoprecipitation (RIP) method (refer to FIG. 1B) and OVA protein was confirmed using the anti-OVA (Sigma). The expression of pTV-OVA and pTV-GE was confirmed by Western Blot Analysis (Lee AH et al., *Vaccine*, 17:473)

<u>EXAMPLE 2: Increase of HA-specific antigen and CTL responses by coimmunization with NP DNA</u>

To analyze the antigen-specific response and CTL response upon coimmunization by simultaneous administration of HA DNA vaccine and NP DNA

vaccine, as prepared in EXAMPLE 1, the inventors of the present invention immunized mice (BALB/c) with one of the mixtures of NP DNA + HA DNA, NP DNA + vector, or HA DNA + (empty) vector two times at 4-week intervals, then took blood from the mice in each group at 4 weeks after the final immunization to measure the anti-NP response and anti-HA response.

In examples of the present invention, 6 to 7-week-old female mice of BALB/c or C57BL/6 character were used (4 to 5-week-old when purchased from B & K Universal Inc.). For DNA immunization of these mice, pTV-NP (50 μg), pTV-HA (50 μg), pTV-gDs-E2t (50 μg), or pTV-GE (50 μg) was dissolved in 100 μl of PBS (phosphate buffered saline) and the resulting solution was intramuscularly injected into the tibialis muscle in both legs of BALB/c mice, 50 μl in each leg. Four weeks after the first immunization, a booster immunization was performed at the same regions with the same DNA mixture.

ELISA for detection of the anti-NP antibody response and anti-HA antibody response was conducted by the known method (Sin JI, *Vaccine*, 15:1827). HA and NP proteins were partially purified from an Influenza bulk vaccine solution (LG chemical Co. Ltd.) using Con-A Cephalos (Pharmacia) column according to the manufacturer's instruction, then each protein solution was separated by SDS-PAGE. Gels corresponding to NP and HA protein bands were cut out and each protein was obtained by the electroelution method. The purified NP protein and HA protein were diluted in PBS to 2 μg/ml, then 50 μl of the resulting protein solution was coated on ELISA plates for analysis.

The analysis result showed that NP DNA + vector immunization and NP DNA + HA DNA immunization were not significantly different in anti-NP response ($p > 0.2$) (refer to FIG. 2A). In contrast, the analysis result also showed that the anti-HA response in the NP DNA + HA DNA-immunized mice increased significantly ($p < 0.05$), as compared with the HA DNA + vector-immunized mice.

Moreover, the inventors of the present invention immunized mice with NP DNA + HA DNA two times at 4-week intervals and measured the NP-specific CTL and HA-specific CTL responses.

Specifically, 4 weeks after the booster immunization, splenocytes of mice in each group were maintained in a CTL analysis culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM b-mercaptoethanol, and 10U/ml recombinant murine IL-2). To stimulate NP-specific and HA-specific lymphocytes, NP peptide as described in Sequence No. 3 and HA peptide as described in Sequence No. 4, cells were adjusted to 7 μM, then stimulated at 37°C in a CO_2 incubator for 6 days. P815(H2d) target cells were pulsed with 5 μM of NP or HA peptide, followed by labeling with 51Cr, then reacted with the stimulated effector cell to measure cytotoxicity.

The above result showed that the NP-specific CTL response was not affected by coimmunization with NP DNA + HA DNA, but the HA-specific CTL response increased more than 100% (from 15% to 33% lysis) by coimmunization with NP DNA + HA DNA (refer to FIG. 2B). In contrast, the NP-specific CTL and HA-specific CTL responses were not observed in HA DNA-immunized mice and NP DNA-immunized mice, respectively (less than 5% lysis).

From the above result, it can be understood that the coimmunization with NP DNA increases the antibody immune response induced by another DNA vaccine, which is administered together with NP DNA, without changing the NP-specific antibody response induced by NP DNA *per se*.

<u>EXAMPLE 3: Analysis of effect of NP DNA on antibody response and CTL response to other viral antigens</u>

To confirm whether the adjuvant effect of NP DNA, i.e., the phenomenon that

coimmunization with NP DNA and another DNA vaccine increases the immune response to the latter, also occurs upon immunization with other viruses than HA DNA, the inventors of the present invention immunized Balb/c mice with HIV env DNA (pTV-GE) or HCV E2 DNA (pTV-gDs-E2t) and NP DNA 2 times at 4-week intervals to measure the NP-specific antibody response and CTL response, the env-specific antibody response and CTL response, and the E2-specific antibody response and CTL response, respectively.

Specifically, immunization of mice was conducted in the same manner as EXAMPLE 2, and for administration of HA DNA, env DNA, E2 DNA or NP DNA, the mixture of 50 μg of each DNA vaccine (pTV-HA, pTV-GE, pTV-gDs-E2t) and 50 μg of vector (pTV) was used. For measurement of the HIV env-specific CTL response, the splenocytes of non-immunized BALB/c mice were infected with a recombinant vaccine virus (rVV-env, NIH), expressing HIV env, to prepare stimulator cells and the stimulator cells were cultivated for 6 days together with the splenocytes of immunized mice. Then, they were reacted with P815 cells which had been transfected with 20 moi of rVV-env and labeled with 51Cr. Measurement of the HCV E2-specific CLT response was conducted following the method of Song et al. (Song et al., *J. Virol.* 74:2920) and CT26-hgh-E2 was used as stimulator cells and target cells.

As a result, the NP-specific antibody response to NP DNA + vector immunization did not have any difference from those to NP DNA + E2 DNA immunization and NP DNA + HIV env DNA immunization (refer to FIGS. 3A and 3B). However, the HIV-1-specific antibody response was increased by coimmunization with NP DNA and, particularly, formation of the E2-specific antibody by E2 DNA + NP DNA immunization was greatly increased, showing a significant difference from the HIV-1-specific antibody response and E2-specific antibody formation in the E2 DNA + vector group ($p < 0.05$).

Meanwhile, NP CTL response induced by NP DNA + vector immunization did

not have any difference from those induced by NP DNA + E2 DNA immunization and NP DNA + HIV env DNA immunization. However, communization with HIV env DNA or HCV E2 DNA and NP DNA increased the env-specific CTL response (from 19% to 51% lysis) or E2-specific CTL response (from 33% to 48% lysis), as compared with the cases of not using NP DNA (refer to FIGS. 3C and 3D).

The above result indicates that NP DNA can also be used as immune response adjuvants for DNA vaccines against other types of viruses, such as HIV and HCV, as well as influenza vaccine.

EXAMPLE 4: Enhancement of IFN-γ secretion from HA-stimulated lymphocytes of mice immunized with NP DNA + HA DNA

Since the intensity of an antigen-specific Th-1 response can be deduced by measuring the concentration of IFN-γ secreted from lymphocytes, the inventors of the present invention stimulated the splenocytes of mice with HA protein or NP protein at 4 weeks after the final immunization to measure the concentration of IFN-γ.

Specifically, splenocytes isolated from the DNA-immunized mice were maintained in an analysis culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM b-mercaptoethanol). 2 x 10^5 lymphocytes per well were put on 96-well round-bottomed plates, then stimulated at 37°C in CO_2 incubator by NP protein or HA protein which was added to the final concentration of 5 μg/ml. After 4 days, the concentration of IFN-γ in the supernatant was measured using a kit (murine IFN-γ ELISA kit; Pharmingen).

The analysis showed that the concentration of IFN-γ, having been secreted from the lymphocytes of mice immunized with NP DNA + HA DNA by stimulation with NP protein, was not highly different from that secreted from the lymphocytes of mice immunized with NP DNA + vector (307 pg/ml, 352 pg/ml, respectively); however, the

amount of IFN-γ secreted by stimulation with HA protein was 590 pg/ml in the mice immunized with NP DNA + HA DNA, which is 2 times greater than the amount in the mice immunized with HA DNA + vector (285 pg/ml, refer to 4). This means that the coimmunization with NP DNA can increase the HA-specific Th-1 response as well as the HA-specific antibody response and CTL response. In contrast, stimulating the lymphocytes of control DNA-immunized mice with influenza protein, stimulating the lymphocytes of NP DNA + vector-immunized mice with HA protein, and stimulating the lymphocytes of HA DNA + vector-immunized mice with NP protein, respectively, induced IFN-γ secretion of less than 60 pg/ml, which was similar to the amount of IFN-γ secreted in the case of not adding any antigen (media control).

EXAMPLE 5: Analysis of OVA-specific IFN-γ producing T cells by coimmunization with NP DNA + OVA DNA

To confirm whether the immune response adjuvant effect of NP DNA also occurs in using mice of other character or using other types of antigens than HA, Env and E2t, the inventors of the present invention injected OVA DNA into C57BL/6 mice to investigate CD4+ and CD8+ T cell responses to OVA. For this, OVA DNA, OVA DNA + NP DNA, or NP DNA was injected into C57BL/6 mice and, after 4 weeks, quantitative IFN- ELISPOT analysis was performed using I-Ab-restricted OVA peptide, H-2b-restricted OVA, or NP peptide.

For coimmunization with OVA DNA and NP DNA, 50 μg of pTV-NP and 50 μg of pTV-OVA were dissolved in 100 μl of PBS and the resulting solution was intramuscularly injected one time into the tibialis muscles in both legs of C57BL/6 mice by 50 μl. For immunization of NP DNA or OVA DNA alone, the mixture of 50 μg of each DNA vaccine and 50 μg of empty vector (pTV2) was used in the same manner as the above.

5 μg/ml of anti-IFN capture Ab (BD Pharmingen) was treated at 4°C in nitrocellulose-based 96-well plates (Millipore) for 1 day and then blocking-treated in a RPMI culture medium (10% FBS) for 2 hours. Single cell solution was obtained from the splenocyte of OVA DNA-injected mice and was diluted to 1/2. 4×10^5, 2×10^5 and 1×10^5 cells were put on the plates, in triplicate, respectively, and treated with 10 μM of OVA 257-264 or OVA 323-339 peptide, then cultivated at 37°C in a CO_2 incubator for 1 day. After cultivation, the plates were washed 5 times with PBS (0.05% Tween 20) and treated with 2.5 μg/ml of biotin-conjugated anti-IFN detection Ab (BD Pharmingen) at RT (room temperature) for 2 hours, then washed 6 times with PBST. Streptavidin-conjugated alkaline phosphatase, as having been diluted to 1/2,000 with PBST, was treated at RT for 1 hour. Thereafter, a BCIP/NBT was added into the plates to react at RT for about 15 minutes. When blue spots were observed after several hours, the reaction was terminated with excess water. After the plates were dried at RT, the number of spots was counted using the optical microscope.

As expected, a high proportion of IFN-γ producing T cells to the H-2b-restricted NP peptide was observed in a group of NP DNA-injected mice and group of NP DNA + OVA DNA-injected mice, and the above two groups showed similar levels of induction (refer to FIG. 5A). Meanwhile, IFN-γ producing T cells to the H-2b-restricted OVA peptide were formed by injection of OVA DNA, and in co-injection of NP DNA, such phenomenon was enhanced by about 4 – 5 times (refer to FIG. 5B). Similarly, the NP DNA + OVA DNA injection group showed about a 3 – 4 times higher frequency of the I-Ab-restricted OVA NP peptide-specific IFN-γ producing T cells than did the OVA DNA injection group (refer to FIG. 5C). In contrast, upon use of NP peptide, specific IFN-γ producing T cells were rarely observed in the OVA DNA injection group and, in use of OVA peptide, they were rarely observed in the NP DNA injection group, which suggests that the above response is an antigen response specific to an injected DNA.

The above result indicates that the adjuvant effect of NP DNA can be applied,

regardless of the character of mice, to a relatively extensive range of coimmunized DNA antigens to increase CD4 and CD8 T cell responses.

EXAMPLE 6: Analysis of CD8 or CD4 T cell proliferation response by NP DNA + OVA DNA

To observe *in vivo* whether NP DNA affects CD8 or CD4 T cell proliferation in response to other co-injected antigens, the inventors of the present invention used respectively OT-I and OT-II cells, as CD8 and CD4 T cells specific to OVA, to analyze CD8 or CD4 T cell proliferation response.

Specifically, TCR (T Cell Receptor) transgenic OT-I or OT-II mice (Kurts C et al., *J. Exp. Med.* 188:409) were used that were restricted to H-2b or I-Ab and specific to OVA (ovalbumin) epitope 257-264 or 323-339 region. Single cell suspension was obtained from the lymph nodes of 6-week-old OT-I (or OT-II) mice, and treated with anti-HSA (J11d), anti-B220, anti-MHC class II, and anti-CD4 (or anti-CD8) single antibodies at 4°C for 30 minutes, then treated with a rabbit complement at 37°C for 45 minutes to obtain OT-I (or OT-II) cells at a purify of more than 95%. The separated OT-I (or OT-II) cells were diluted with PBS to a concentration of 2 x 10^7 cells/ml, and treated with 5 uM of CFSE (Carboxyfluorescein diacetate succinimidyl ester) at 37°C for 10 minutes so as to trace the cell division *in vivo*, thereby obtaining CFSE-labeled OT-I (or OT-II) cells. To detect whether the cell division of a specific cell occurs *in vivo*, the 2 x 10^6 cells were labeled with CFSE fluorescent material and then injected into the blood vessel of 6 to 7-week-old female C57BL/6 mice. After 1 day, NP DNA + OVA DNA, OVA DNA, or NP DNA was intramuscularly injected into the mice and, after 9 days, the lymph node drainage of each mouse was separated to analyze whether the cell division of the CFSE-labeled OT-I or OT-II cell occurred using a lacticiferous cell flow cytometry. For analysis of the lacticiferous cell, the lymph nodes of popliteal and inguinal were separated from the OVA DNA-injected mice to obtain single cell

suspension. The single cell suspension was treated with PerCP-conjugated anti-CD4 or CD8 mAb and PE-conjugated V2 mAb at 4°C for 15 minutes, then 50,000 – 100,000 cells were collected using FACScalibur (BD science). The analysis of cell division was performed using the CellQuest software.

As the result of analysis, 5 – 6 times of OT-I cell divisions by OVA DNA injection were observed (refer to FIG. 6B), wherein upon coimmunization with OVA DNA and NP DNA, the rate of OT-I cell division was observed to be even higher (refer to FIG. 6A). Contrast to the OT-I cells, cell division of OT-II cells by OVA DNA injection was almost not observed (refer to FIG. 6B). However, when NP DNA was injected together with OVA DNA, a remarkable increase in cell division of OT-II cell was observed, like in the case of OT-I cells (refer to FIG. 7A). Meanwhile, upon injection of NP DNA alone, OT-I and OT-II cell divisions were not observed (refer to FIGS. 6C and 7C), which means that the OT-I and OT-II cell divisions are an OVA antigen-specific response induced by OVA DNA.

The above result indicates that NP DNA has an effect on CD8 or CD4 T cell response induced by other coimmunized DNA (HA, env, E2 or OVA), injected together with NP DNA, particularly, on the rate/induction of cell division.

EXAMPLE 7: Increase of the initial survival rate after lethal influenza challenge

The inventors of the present invention challenged the mice immunized with the mixture of HA DNA and NP DNA and the mice immunized with HA DNA + vector and NP + vector, respectively, with fifty lethal doses (50 LD_{50}) of influenza A/Jap/57 at 6 weeks after the booster immunization. Specifically, 6 weeks after the booster immunization, mice in each group were anesthetized with an avertin solution and infected with 50 LD_{50} of influenza A/Jap/57 via the intranasal route. The body weight and survival of mice in each group were monitored at a selected date. The mean weight

of mice in each group was calculated by fixing the weight of surviving mice and the weight of dead mice by influenza infection as 0 value and then comparing the weight of mice before infection therewith. Survival and weight change of each mouse were monitored and observed until 20 days after infection.

As a result, 20 days after infection, the NP DNA + HA DNA-immunized mice group exhibited a survival rate of 42%, which is similar to or rather less than those of NP DNA + vector-immunized and HA DNA + vector-immunized mice groups, 50% and 45%, respectively ($p > 0.2$). However, between 4 – 8 days after injection, the survival rate of NP DNA + HA DNA-immunized mice group was higher than those of NP DNA + vector-immunized and HA DNA + vector-immunized mice group (refer to FIG. 8A). Moreover, during this period, the mean body weight of NP DNA + HA DNA-immunized mice group was also higher than those of any other groups (refer to FIG. 8B).

INDUSTRIAL APPLICABILITY

As described above, according to the method of the present invention, an influenza NP gene DNA is used as an adjuvant for a DNA vaccine to enhance the immune response of the DNA vaccine. Accordingly, this method can be used for effective prevention against or treatment of influenza, AIDS, hepatitis B, hepatitis C, cancer, tuberculosis, malaria, etc. and provide the information regarding the development of influenza vaccines. Furthermore, this method can help research into immune response adjuvants of AIDS and hepatitis, and research for effect enhancement of a vaccine comprising more than two DNA components. In addition, the system used in the present invention can be applied to the research models for immune interference or enhancement in DNA immunizations with multiple-components.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : SUNG, Jae Kap
LGCI R&D park,
#104-1, Moonji-dong, Yuseong-gu, Daejeon 305-380,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli **XL1-blue/pTV-NP**	**KCTC 10193BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **February 27 2002.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **Korean Collection for Type Cultures**	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Bae K S. BAE, Kyung Sook, Director Date: **March 05 2002**

Form BP/4 (KCTC Form 17) sole page

WHAT IS CLAIMED IS:

1. A method for enhancing an immune response of a DNA vaccine by administering an influenza NP gene DNA together with said DNA vaccine.

2. The method according to claim 1, wherein said influenza NP gene DNA is DNA having the full nucleotide sequence or more than 50% of a nucleotide sequence for encoding a protein having more than 90% amino acid sequence homology to an influenza NP protein.

3. The method according to claim 2, wherein said DNA is DNA having the nucleotide sequence described in Sequence No. 1.

4. The method according to claim 1, wherein said influenza NP gene DNA is administered with the influenza NP gene DNA inserted into an expression vector, the expression vector being a vector having the transcription promotor selected from a group consisting of CMV (cytomegalovirus) promotor, RSV (Rous sarcoma virus) promotor, β-actin promotor, SV40 (simian virus 40) promotor and muscle creatine kinase promotor, and the transcription terminator selected from a group consisting of SV40 poly(A) and BGH terminator.

5. The method according to claim 4, wherein a transformant, obtained by inserting said influenza NP gene into said expression vector, is pTV-NP (Accession No. KCTC 10193BP).

6. The method according to claim 1, wherein said DNA vaccine is a DNA vaccine against one or more immune antigens selected from a group consisting of influenza, varicella virus, diphtheria, tetanus, polio virus, malaria, herpes virus, HIV, papilloma virus, hepatitis B virus, hepatitis C virus, rotavirus, cholera, measles and tuberculosis.

7. An adjuvant for enhancing immune response to a DNA vaccine, comprising an

influenza NP gene DNA.

8. A vaccine composition comprising an influenza NP gene DNA and a DNA vaccine against an immune antigen.

9. The composition according to claim 8, wherein said influenza NP gene DNA is DNA having the full nucleotide sequence or more than 50% of the nucleotide sequence for encoding a protein having more than 90% amino acid sequence homology to an influenza NP protein.

10. The composition according to claim 9, wherein said DNA is DNA having the nucleotide sequence described in Sequence No. 1.

11. The composition according to claim 8, wherein said influenza NP gene DNA is administered with the influenza NP gene DNA inserted into an expression vector, the expression vector being a vector having the transcription promotor selected from a group consisting of CMV (cytomegalovirus) promotor, RSV (Rous sarcoma virus) promotor, β-actin promotor, SV40 (simian virus 40) promotor and muscle creatine kinase promotor, and the transcription terminator selected from a group consisting of SV40 poly(A) and BGH terminator.

12. The composition according to claim 11, wherein a transformant, obtained by inserting said influenza NP gene into said expression vector, is pTV-NP (Accession No. KCTC 10193BP).

13. The composition according to claim 8, wherein said DNA vaccine is a DNA vaccine against one or more immune antigens selected from a group consisting of influenza, varicella virus, diphtheria, tetanus, polio virus, malaria, herpes virus, HIV, papilloma virus, hepatitis B virus, hepatitis C virus, rotavirus, cholera, measles and tuberculosis.

DRAWINGS

FIG. 1

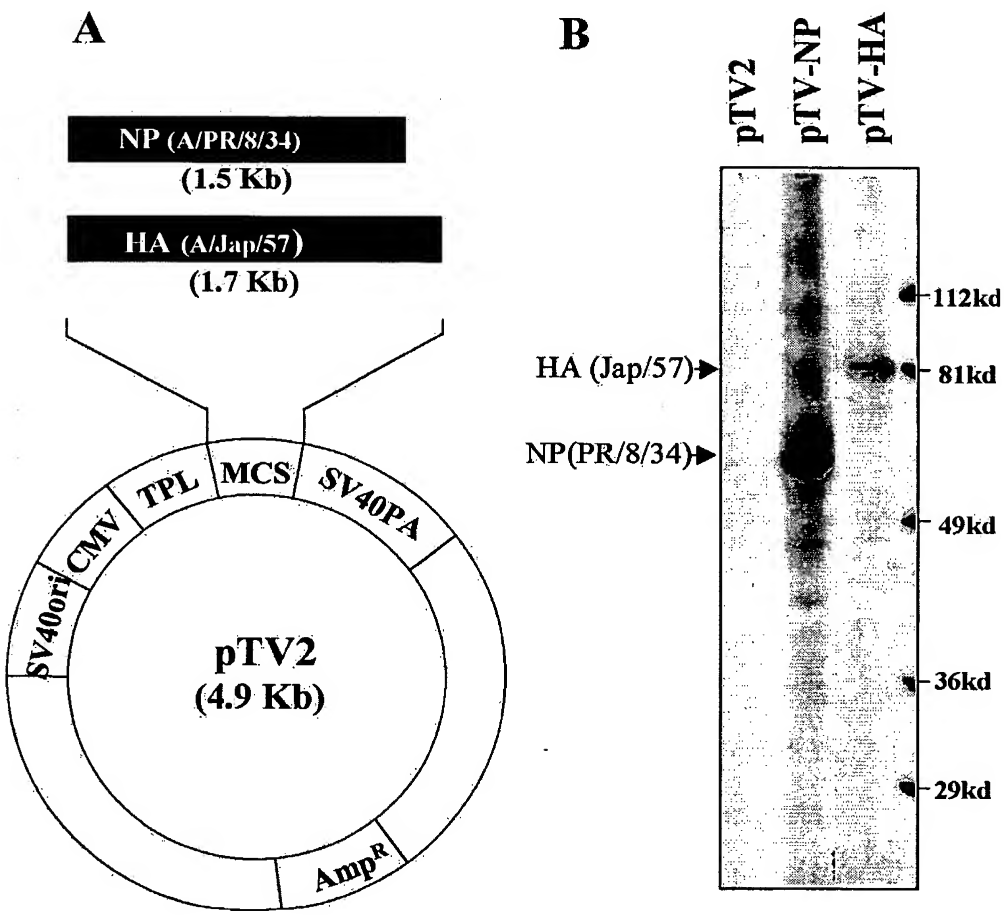


FIG. 2

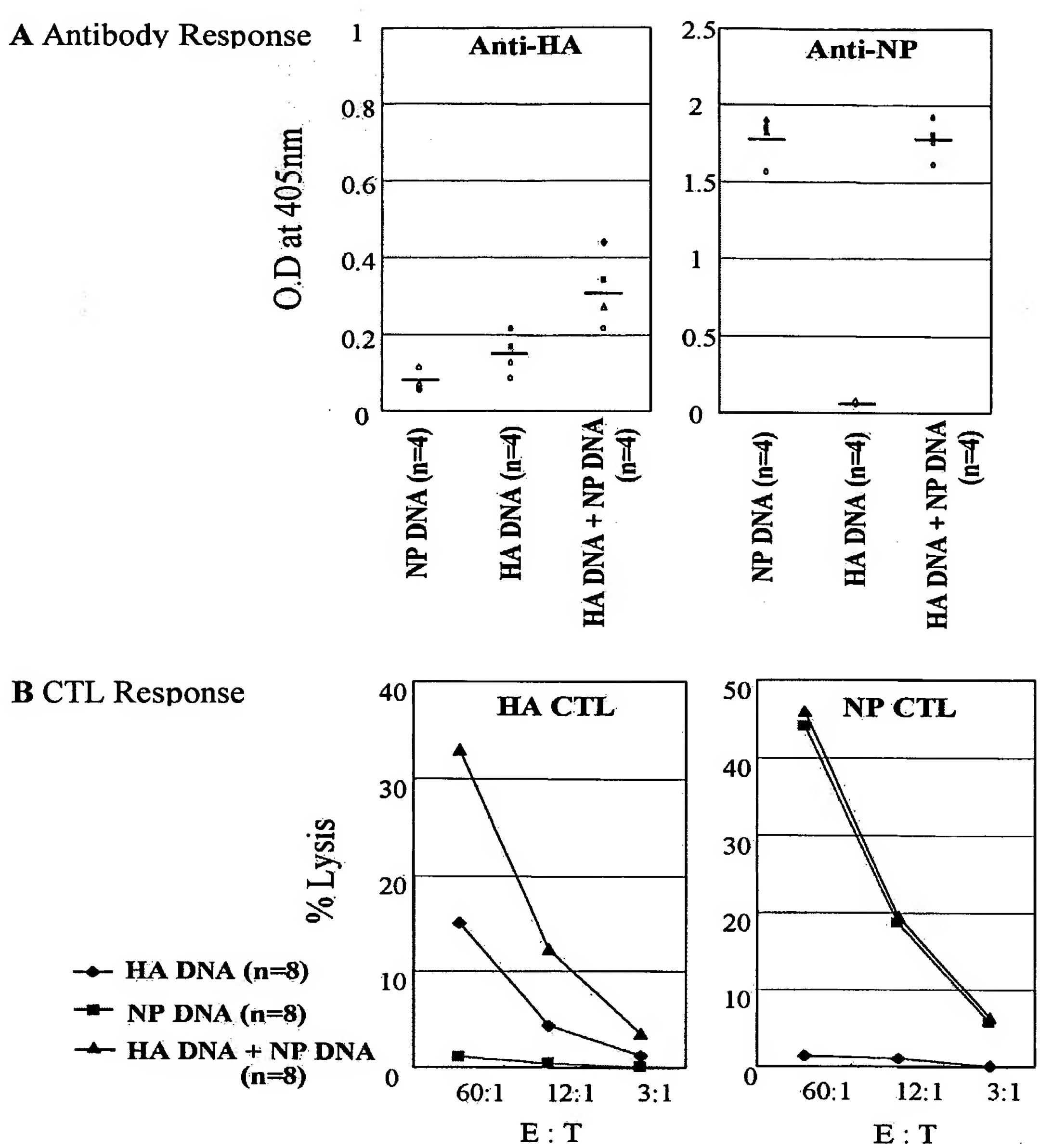
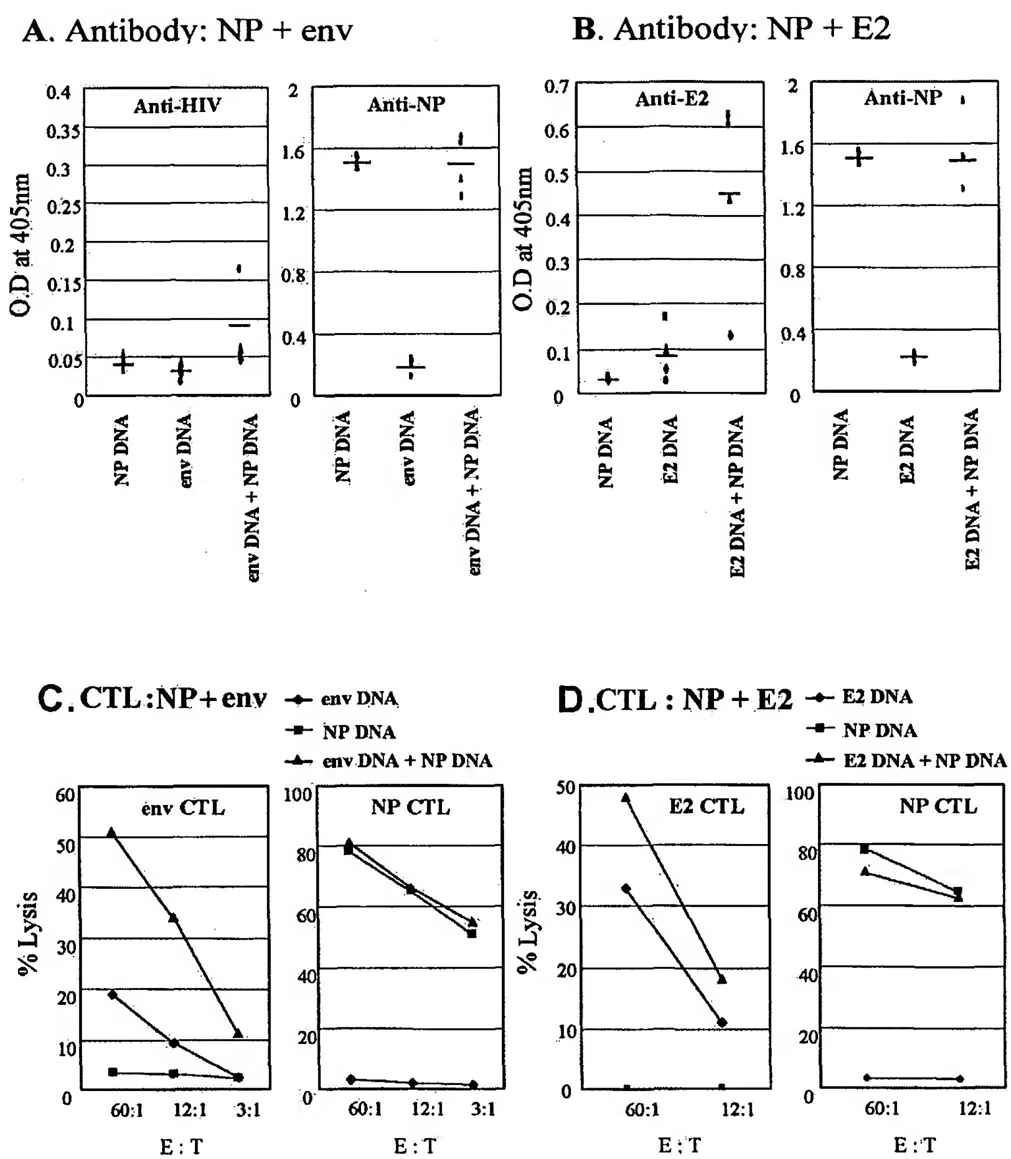

A Antibody Response
O.D at 405nm
Anti-HA
Anti-NP
1
0.8
0.6
0.4
0.2
0
2.5
2
1.5
1
0.5
0
NP DNA (n=4)
HA DNA (n=4)
HA DNA + NP DNA (n=4)
B CTL Response
% Lysis
HA CTL
NP CTL
40
30
20
10
0
50
40
30
20
10
0
60:1
12:1
3:1
E : T
HA DNA (n=8)
NP DNA (n=8)
HA DNA + NP DNA (n=8)

FIG. 3


A. Antibody: NP + env
B. Antibody: NP + E2
Anti-HIV
Anti-NP
Anti-E2
Anti-NP
O.D at 405nm
NP DNA
env DNA
env DNA + NP DNA
E2 DNA
E2 DNA + NP DNA
C. CTL:NP+env
env DNA
NP DNA
env DNA + NP DNA
D. CTL : NP + E2
E2 DNA
NP DNA
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env CTL
NP CTL
E2 CTL
% Lysis
60:1
12:1
3:1
E : T

FIG. 4

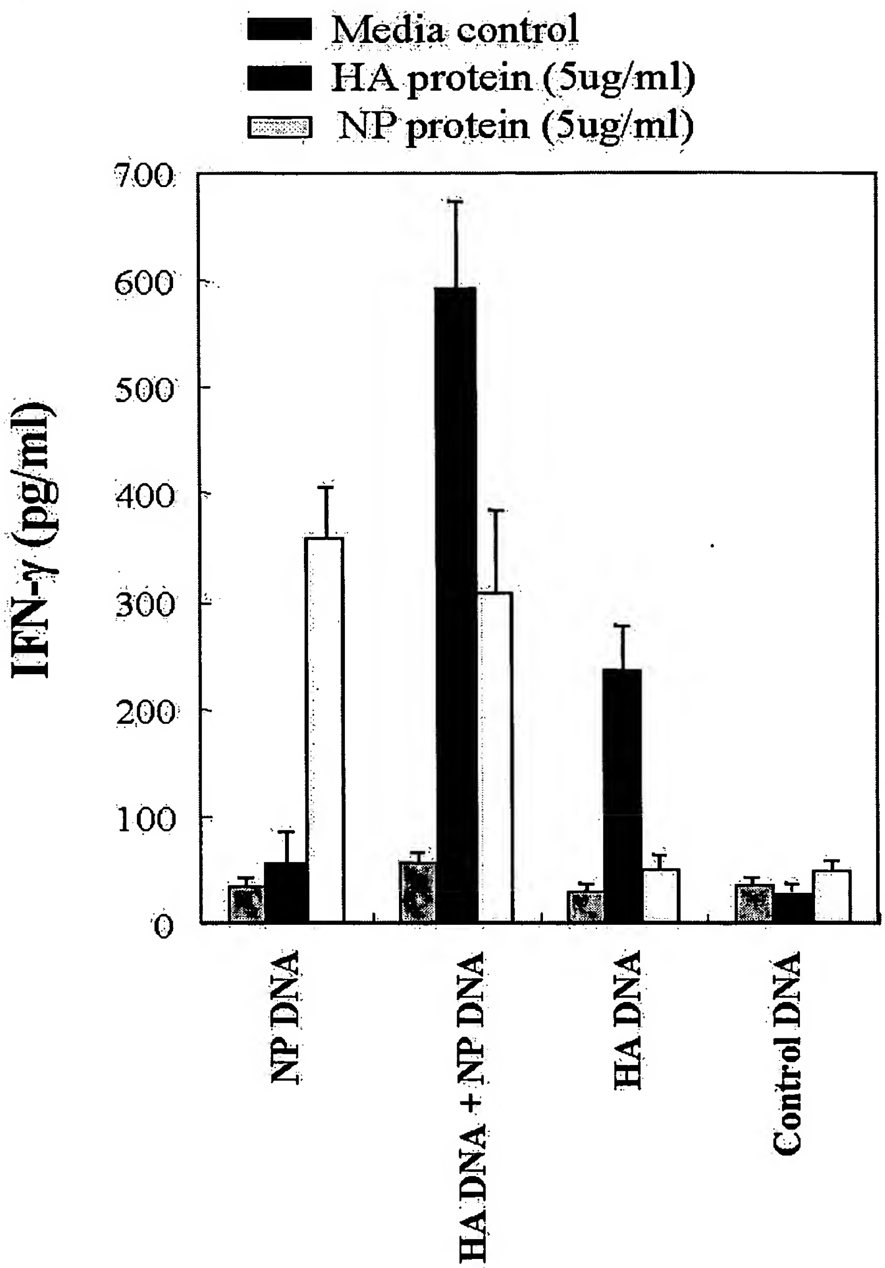

Media control
HA protein (5ug/ml)
NP protein (5ug/ml)
IFN-γ (pg/ml)
700
600
500
400
300
200
100
0
NP DNA
HA DNA + NP DNA
HA DNA
Control DNA

FIG. 5

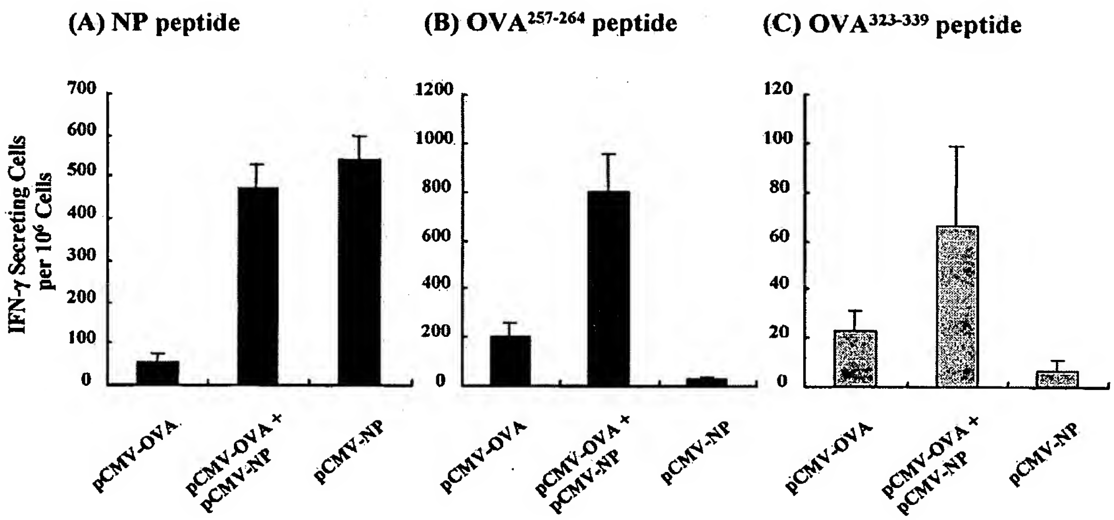

(A) NP peptide
(B) OVA257-264 peptide
(C) OVA323-339 peptide
IFN-γ Secreting Cells per 10^6 Cells
700
600
500
400
300
200
100
0
1200
1000
800
600
400
200
0
120
100
80
60
40
20
0
pCMV-OVA
pCMV-OVA + pCMV-NP
pCMV-NP

FIG. 6

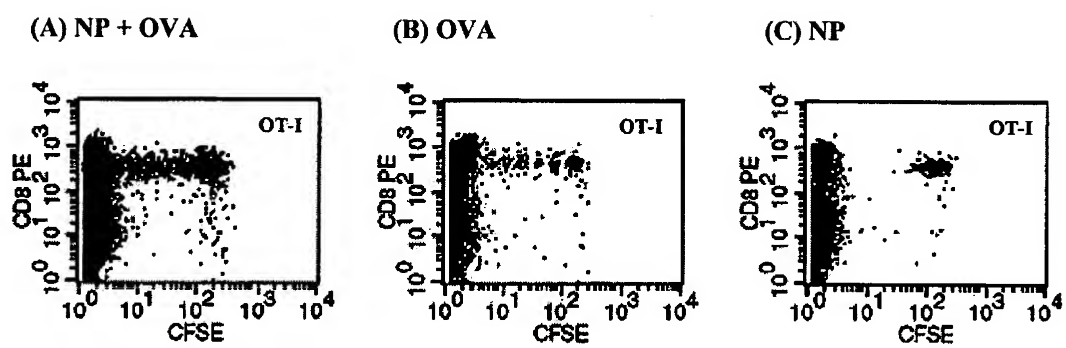

(A) NP + OVA
(B) OVA
(C) NP
OT-I
CD8 PE
CFSE
10^0
10^1
10^2
10^3
10^4

FIG. 7

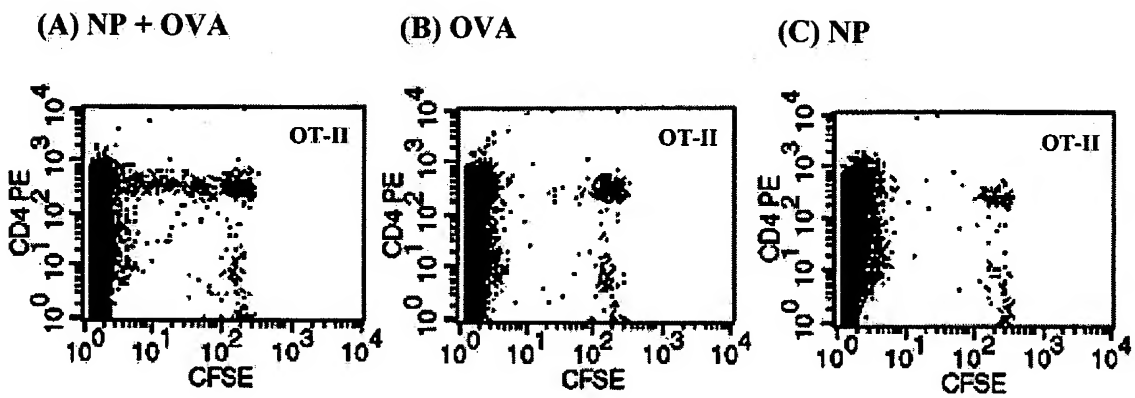
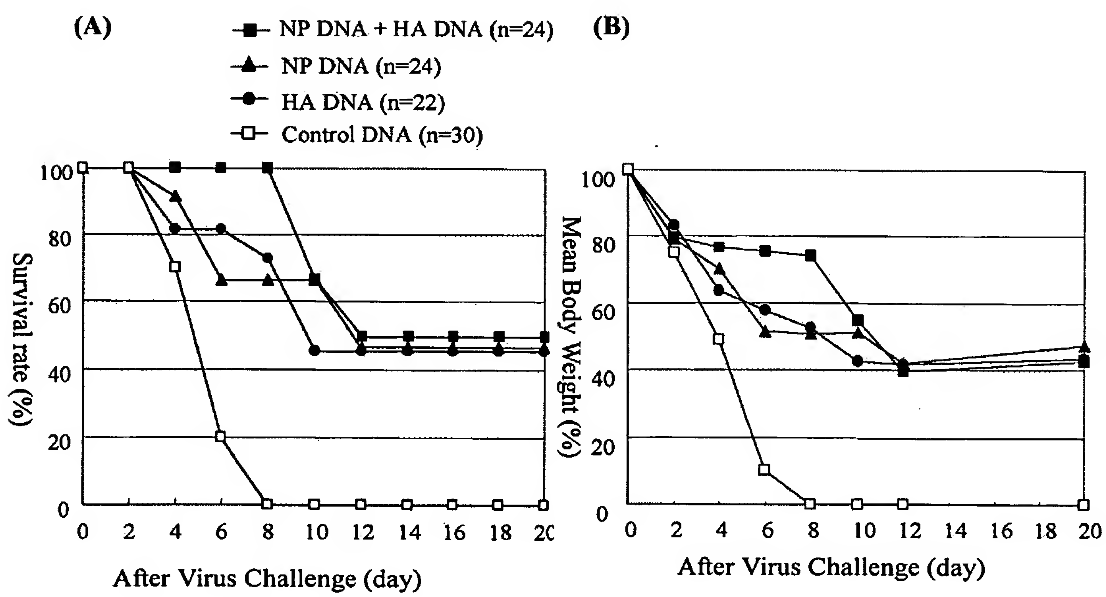

(A) NP + OVA
(B) OVA
(C) NP
OT-II
CD4 PE
CFSE

FIG. 8


(A)
(B)
NP DNA + HA DNA (n=24)
NP DNA (n=24)
HA DNA (n=22)
Control DNA (n=30)
Survival rate (%)
Mean Body Weight (%)
After Virus Challenge (day)

Sequence Listing

<110> LG Life Sciences Ltd.

<120> Method for enhancing immune responses by codelivering influenza NP DNA in DNA immunization

<130> LL03PCT002

<150> KR 10-2002-0013574
<151> 2002-03-13

<160> 4

<170> KopatentIn 1.71

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<211> 1497
<212> DNA
<213> Influenza A/PR/8/34 (H1N1) nucleoprotein

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atccaaatgt gcaccgaact caaactcagt gattatgagg gacggttgat ccaaaacagc 180

ttaacaatag agagaatggt gctctctgct tttgacgaaa ggagaaataa ataccttgaa 240

gaacatccca gtgcggggaa agatcctaag aaaactggag gacctatata caggagagta 300

aacggaaagt ggatgagaga actcatcctt tatgacaaag aagaaataag gcgaatctgg 360

cgccaagcta ataatggtga cgatgcaacg gctggtctga ctcacatgat gatctggcat 420

tccaatttga atgatgcaac ttatcagagg acaagagctc ttgttcgcac cggaatggat 480

cccaggatgt gctctctgat gcaaggttca actctcccta ggaggtctgg agccgcaggt 540

gctgcagtca aaggagttgg aacaatggtg atggaattgg tcagaatgat caaacgtggg 600

Sequence Listing

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caagtgagag agagccggaa cccagggaat gctgagttcg aagatctcac ttttctagca 780

cggtctgcac tcatattgag agggtcggtt gctcacaagt cctgcctgcc tgcctgtgtg 840

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atagaccctt tcagactgct tcaaaacagc caagtgtaca gcctaatcag accaaatgag 960

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gcatctgcgg gccaaatcag catacaacct acgttctcag tacagagaaa tctccctttt 1260

gacagaacaa ccgttatggc agcattcagt gggaatacag aggggagaac atctgacatg 1320

aggaccgaaa tcataaggat gatggaaagt gcaagaccag aagatgtgtc tttccagggg 1380

cggggagtct tcgagctctc ggacgaaaag gcagcgagcc cgatcgtgcc ttcctttgac 1440

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Sequence Listing

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gaatgtgata ggcttctaag tgtgccagaa tggtcctata taatggagaa agaaaacccg 300

agagacggtt tgtgttatcc aggcagcttc aatgattatg aagaattgaa acatctcctc 360

agcagcgtga aacatttcga gaaagtaaag attctgccca aagatagatg gacacagcat 420

acaacaactg gaggttcacg ggcctgcgcg gtgtctggta atccatcatt cttcaggaac 480

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acaagcggag aacaaatgct aataatttgg ggggtgcacc atcccaatga tgagacagaa 600

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Sequence Listing

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<212> PRT
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<223> HA peptide

<400> 4

Sequence Listing

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INTERNATIONAL SEARCH REPORT	International application No. PCT/KR03/00471

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 39/145

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 07 A61K 39/145

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

STN (EMBASE, CA)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AKIKO IWASAKI, et al. 'Enhanced CTL Responses Mediated by Plasmid DNA Immunogens Encoding Costimulatory Molecules and Cytokines', The Journal of Immunology, 1997, Vol.158, No.10, pp.4591-4601. See the whole document	1-13
A	JOHN W. SHIVER, et al., 'Humoral and cellular immunities elicited by DNA vaccines: Application to the human immunodeficiency virus and influenza', Advanced Drug Delivery Reviews, 1996, pp.19-31 See the whole document	1-13
A	SUDHIRDAS K. PRAYAGA, et al.,'Manipulation of HIV-1 gp120-specific immune responses elicited via gene gun-based DNA immunization', Vaccine, 1997, Vol.15, pp.1349-52 See the whole document	1-13
A	LAWRENCE S.D. ANTHONY, et al.,'Priming of CD8+ CTL effector cells in mice by immunization with a stress protein-influenza virus nucleoprotein fusion molecule', Vaccine, 1999, Vol.17, pp.373-83 See the whole document	1-13

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 JUNE 2003 (18.06.2003)	19 JUNE 2003 (19.06.2003)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	KIM, Hee Jin 김희진
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5412

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92/16619 A1 (THE UNITED STATES OF AMERICA) 1 October 1992 See the whole document	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/00471

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-6
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-6 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

1. [ ] As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any addition fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR03/00471

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W09216619A1	01.10.1992	AU1651992A	21.10.1992
		W09216619A1	01.10.1992